

Modelling antisepsis using defined populations of facultative and anaerobic wound pathogens grown in a basally perfused biofilm model

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38 Abstract

39 An *in vitro* model was developed to assess the effects of topical antimicrobials on taxonomically
40 defined wound biofilms. Biofilms were exposed over 7d to povidone-iodine, silver acetate or
41 polyhexamethylene biguanide (PHMB) at concentrations used in wound dressings. The rank order of
42 susceptibility in multi-species biofilms, based on an analysis of the average bacterial counts over
43 time (low to high) was *P. aeruginosa*>Methicillin-resistant *Staphylococcus aureus* (MRSA)>*B.*
44 *fragilis*>*S. pyogenes*. The rank order of effectiveness for the antimicrobials in the biofilm model was
45 povidone-iodine>PHMB>silver acetate. None of the test compounds eradicated *P. aeruginosa* or
46 MRSA from the biofilms although all compounds except silver acetate eliminated *S. pyogenes*.
47 Antimicrobial effectiveness against bacteria grown in multi-species biofilms did not correlate with
48 planktonic susceptibility. Defined biofilm populations of mixed-species wound pathogens could be
49 maintained in the basal perfusion model, facilitating the efficacy testing of treatments regimens and
50 potential dressings against multi-species biofilms composed of wound isolates.

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52
53

Introduction

54 Chronic wounds represent a considerable challenge to wound care professionals and healthcare
55 resources, having a significant bearing upon patient morbidity and mortality (Scali and Kunimoto
56 2013). Whilst the processes influencing the transition to a chronic wound state are complex and
57 depend on host factors such as wound aetiology, co-morbidities and anatomical location, infection
58 and the presence of biofilms have been implicated as important contributors (Roy et al. 2014, Scali
59 and Kunimoto 2013, Schierle et al. 2009). Biofilms have been described as structured microbial
60 communities encased in an exopolymer matrix (Costerton 1999, Hall-Stoodley et al. 2004) that are
61 normally taxonomically diverse and adapted to survive inimical conditions (Gilbert et al. 2002,
62 Watanabe et al. 1998). Evidence suggests that through cellular proximity, biofilm formation can
63 enhance horizontal gene transfer rates and may therefore drive bacterial the transfer of resistance
64 determinants (Hausner and Wuertz 1999, Sorensen et al. 2005) and microbial evolution over longer
65 timescales (as reviewed by Claessen *et al.*, 2014). Through genotypic and phenotypic heterogeneity,
66 biofilms exhibit substantially increased tolerance to antimicrobial therapies and host immune
67 responses (Gilbert et al. 2002, Pedersen 1992, Stewart and Costerton 2001).

68

69 The association of biofilms with chronic wounds (Bjarnsholt et al. 2008, Kanno et al. 2009, Neut et
70 al. 2011, Roy et al. 2014, Wolcott and Rhoads 2008) has driven the development and application of
71 antimicrobial dressings based on their effectiveness against the most recalcitrant forms of microbial
72 growth present in this environment, which includes organisms displaying biochemical resistance,
73 such as *Pseudomonas* spp. (Ramos et al. 2010, Rochex and Lebeault 2007, Walters et al. 2003),
74 Methicillin resistant *Staphylococcus aureus* (MRSA) (McCarthy et al. 2015, Ohadian Moghadam et
75 al. 2014) and microbial biofilms, that generally exhibit greater tolerance to antimicrobials than their
76 planktonic counterparts regardless of taxonomic composition (Gilbert et al. 2002).

77 A variety of antimicrobial wound dressings are available incorporating active compounds such as
78 iodine and silver that are designed with broad-spectrum antimicrobial activity in mind (Barnea et al.
79 2010, Bradley et al. 1999, Butcher 2012). *In vitro* models supporting the growth of biofilm
80 communities (Oates and McBain 2016) can be used in the preclinical investigation of antimicrobial
81 dressings for their potential effectiveness against wound bacteria (Humphreys et al. 2011, Werthén et
82 al. 2010, Wilkinson et al. 2016, Woods et al. 2012). Several models have been developed specifically
83 for investigating wound-associated biofilms, including the Lubbock Chronic Wound Biofilm, which
84 is a closed, static media system (Sun et al. 2008), a flat-bed perfusion model that comprises a
85 continuous media flow system with an inert substratum (Thorn and Greenman 2009), and the
86 modified drip-slide reactor (Lipp et al. 2010). Various other continuous culture systems have also
87 been developed for biofilm applications including the Constant Depth Film Fermenter (CDFF) and
88 devices housing Sorbarods (cylindrical cellulose filters). These models have been used for a range of
89 applications including wound biofilm investigations (Hill et al. 2010, Hodgson et al. 1995). Axenic
90 or combined cultures of bacteria of relevance to wounds such as *Staphylococcus aureus* and
91 *Pseudomonas aeruginosa* have been utilised in *in vitro* models in several previous investigations
92 (Oates and McBain 2016, Thorn and Greenman 2009, Werthén, Henriksson, Jensen, Sternberg,
93 Givskov and Bjarnsholt 2010) and can generate robust and reproducible data, allowing for example,
94 the examination of axenic biofilm growth dynamics and responses to antimicrobials. Defined
95 community systems where a small number of culturable bacteria are grown together in a biofilm
96 model are however, likely to be more representative of the communities which they aim to represent
97 as opposed to single species systems (Thorn and Greenman 2009).

98

99 In the current investigation, a model in which the effectiveness of topical antimicrobials can be
100 assessed against four functionally distinct wound pathogens grown together in biofilms was
101 developed. The system has several features of use for preclinical evaluation of antimicrobials for

102 application to wounds since biofilms grown in the system are fed basally like the nutrient supply in
103 chronic wounds, and it supports the growth of obligate aerobic, anaerobic and facultative bacterial
104 organisms, enabling assessment of the differential effects of distinct classes of antimicrobial against
105 broad physiological groups of wound pathogens.

106

107 **Methods**

108 ***Bacteria***

109 Four bacteria of significance to wound infections and with variable growth requirements were
110 selected for inclusion in the defined biofilm consortium. *Pseudomonas aeruginosa* and
111 *Streptococcus pyogenes* were isolated from infected wounds. Methicillin resistant *Staphylococcus*
112 *aureus* (MRSA) NCTC 11939 and *Bacteriodes fragilis* NCTC 9343 were obtained from Public
113 health England Southampton, UK.

114

115 ***Chemicals and media***

116 Vantocil (a 20% [vol/vol] aqueous solution of polyhexamethylene biguanide) was obtained from
117 Arch Biocides, Inc. (Manchester, United Kingdom). Dehydrated bacteriological media were obtained
118 from Oxoid (Basingstoke, Hampshire, U.K.) and reconstituted per instructions supplied by the
119 manufacturer. Unless otherwise stated all other chemicals used were supplied by Sigma (Poole,
120 Dorset, U.K.).

121

122 **Model Development and Optimisation**

123 ***Relative fitness assays***

124 To assess whether the four bacterial strains selected for the mixed wound consortium could grow
125 stably when combined, relative fitness was assessed in pair-wise combinations during growth on
126 5mm x 5mm mono-acetate filters (BullBrand Ltd. Keighthly, UK) using a modified competition

127 assay (Lenski et al. 1991, Rozen et al. 2007), as follows: stationary phase cultures were grown
128 overnight in a simulated wound fluid (SWF) medium consisting of foetal calf serum (49% v/v),
129 Mueller-Hinton broth (49% v/v) and laked horse blood (2% v/v). Axenic cultures were then adjusted
130 to achieve an initial inoculum density of $c. 1 \times 10^8$ CFU/ml and combined in a 1:1 ratio with a
131 partner culture and then further diluted in fresh sterile SWF to give a final density of $c. 1 \times 10^6$
132 CFU/ml. The final combined inoculum was dispensed (1 ml) onto 5mm x 5mm monoacetate filters
133 (n=6) housed within the wells of a microtitre plate. To determine the initial starting CFU/mm³ of
134 each bacterial isolate, three filters were aseptically removed, aseptically sectioned, with sections
135 placed in a plastic Universal bottle (Scientific Laboratory Supplies, Nottingham, UK) containing 9
136 ml of half-strength Thioglycolate Broth and 5 mm sterile glass beads (n = 5) (Merck, Darmstadt,
137 Germany). To ensure uniform distribution of cells throughout the diluent, Universals were vortexed
138 for 10 sec., serially diluted in half strength thioglycolate broth and plated onto suitable selective
139 agars, as follows: Mannitol Salt Agar, Pseudomonas CFC selective agar, G-N Anaerobe Selective
140 Agar and Streptococcus Selective Agar. The remaining filters were incubated for 48 h and then
141 viable counts were performed to determine the endpoint CFU/mm³.

142

143 ***Basal perfusion wound biofilm models***

144 A diagram of the basal perfusion model is shown in Figure 1 and 2. Briefly, the system consists of a
145 hollow cylinder Pyrex glass outer casing (8 cm Ø x 4.5 cm depth) housing three machined
146 polytetrafluoroethylene (PTFE) components comprising a lid, a central receptacle and a waste
147 reservoir. The lid measures $c. 8$ cm Ø and contains a central inlet tube to deliver media to the central
148 receptacle. The central receptacle is machined from PTFE and contains a central fluid basin (2.2 cm
149 Ø x 3 cm), which is fed with media via the lid inlet tube (Figure 1). There are 8 channels that lead
150 away from the central fluid basin towards 8 individual filter receptacles (1 cm Ø) where each filter
151 resides. Each filter receptacle incorporates a lower fluid bulk section and an upper level receptacle

152 separated by a small PTFE rim (0.5 cm Ø) upon which 5mm x 5mm mono-acetate filters (Bullbrand
153 Ltd. Keighthly, UK) are placed. Medium fed into the central fluid basin is distributed into the lower
154 fluid bulk section of the filter receptacles via the channels shown in Figure 1. The fibres of the 5mm
155 x 5mm mono-acetate filter act as a wick, drawing media up from the bulk fluid, saturating the filter.
156 Drainage channels leading away from the filter receptacles allow the removal of excess media and
157 waste products into the waste reservoir, where it is drained from the whole system via the outlet tube
158 in the Pyrex glass casing. Temperature (37°C) and atmosphere (O₂) were maintained by placing the
159 device in an aerobic incubator. SWF was continually supplied to the device at a rate of *c.* 1.5 ± 0.1
160 ml h⁻¹ by peristaltic pump (Minipulse 3, Gilson, Villiers-Le-Bel, France). Once inoculated with all
161 four bacteria, the model was run for up to 8 days.

162

163 ***Inoculation and population stability assays***

164 Initial trials into the inoculation and maintenance of the defined wound consortium (MRSA, *S.*
165 *pyogenes*, *P. aeruginosa* and *B. fragilis*) determined that a staggered inoculation process was
166 required (data not shown). Prior to inoculation, the system was preconditioned with SWF for 1 h.
167 Stationary phase axenic cultures of MRSA and *S. pyogenes* were each adjusted to give an inoculum
168 density of *c.* 1 x 10⁸ CFU/ml, combined in a 1:1 ratio and a further diluted to give cellular density of
169 each organism of *c.* 1 x 10⁷ CFU/ml. Filters were inoculated in situ with 1 ml of the dual species
170 inoculum. The basal perfusion model was continuously fed at a rate of 1.5 ± 0.1 ml h⁻¹ by peristaltic
171 pump. After 24 h, stationary phase cultures of *P. aeruginosa* and *B. fragilis* were similarly adjusted
172 and combined to give a density of *c.* 1 x 10⁷ CFU/ml of each organism and 1 ml of this mixed
173 inoculum was used to inoculate each filter. The model was incubated for a further 24 h under
174 continuous culture conditions before sampling. Filters were then removed every 24 h and placed in 9
175 ml of half strength thioglycolate broth containing 5 mm sterile glass beads (n=5) (Merck, Darmstadt,
176 Germany), vortexed for 10 s, serially diluted in half strength thioglycolate broth and plated onto

177 suitable selective agar as follows to allow for viable counts of each distinct organism, as outlined
178 above. All experiments were undertaken independently, in triplicate.

179

180 ***Homogeneity of population densities across filters***

181 The basal perfusion model was designed to grow wound associated bacterial communities and assess
182 the response to antimicrobial therapies over time. To ensure that the population densities in each
183 filter were homogenous with those in the other filters i.e. a population density of a randomly sampled
184 filter would be approximately the same in another filter within the same model, several independent
185 basal perfusion models were set up as previously described and run to a timed endpoint. Four filters
186 were randomly selected and removed at each end-point and viable counts preformed as previously
187 described. This was continued until viable count data for 4 filters for each individual day over 8 d
188 were completed. Statistical significance was determined using analysis of variance (ANOVA)
189 combined with post-hoc analysis of least significant difference (LSD) test to determine significant
190 difference.

191

192 ***Imaging of biofilms using environmental scanning electron microscopy***

193 Environmental scanning electron microscopy (ESEM) was used to image biofilm communities. A
194 FEI Quanta 200 ESEM was used under a low vacuum (<0.83 torr), permitting interrogation of
195 putative biofilm structures and microcolonies whilst conserving the hydrated state of the sample.
196 Filters were sectioned both transversally and horizontally to ensure that representative images were
197 obtained.

198

199 ***Determination of antimicrobial susceptibility in planktonic culture***

200 The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were
201 determined axenic planktonic populations. Briefly, stationary phase cultures of MRSA, *S. pyogenes*,

202 *P. aeruginosa* and *B. fragilis* grown in SWF, were adjusted in fresh sterile SWF media to give a cell
203 density of *c.* 1×10^6 CFU/ml. Inocula were dispensed (100 μ l) in wells of a flat bottom 96 well
204 microtitre plate within 30 min. of preparation. Stock antimicrobial solutions were prepared in SWF
205 and dispensed (100 μ l) into the first column containing bacterial inocula in the microtitre plate.
206 Doubling dilutions of solutions were then made. Plates were incubated in a wet box at 37°C for 24 h.
207 The MIC endpoint was determined as the lowest concentration of antimicrobial at with no visible
208 growth. The MBC was determined by transferring 10 μ l of inocula (that showed no growth) and spot
209 plating onto agar plates. Agar plates were incubated for 24 h and then examined for colony growth.
210 The MBC endpoint was determined as the lowest concentration that resulted in no visual growth of
211 colonies.

212

213 *Assessment of differential antisepsis in the wound model system*

214 To assess the effect of the selected antiseptic agents on biofilm communities a series of basal
215 perfusion models were set up. Concentrations commonly utilized topically in wound care
216 (Bolton 2006, Burks 1998, Butcher 2012, Fong and Wood 2006, Goldenheim 1993) were used that
217 were in all cases greater than the corresponding MICs of the test bacteria. These were povidone-
218 iodine (10% w/v); polyhexamethylene biguanide (PHMB) (0.5 % v/v) and silver acetate (0.05 %
219 w/v). Once systems were established, one untreated filter was removed on day 1 (baseline) and
220 viable counts performed as previously described. The perfused media were then supplemented with
221 the antimicrobial of interest (added to media to produce the desired concentration) and systems were
222 fed with the antimicrobial dosed SWF at rate of 1.5 ± 0.1 ml h⁻¹ by peristaltic pump for the
223 remainder of the model run. Filters were removed daily, and viable counts performed as previously
224 described.

225

226

227

228 **Statistical Analysis**

229 Data analysis was conducted using a linear mixed-effects model to analyse the average bacterial
230 count over time to answer two questions. The first pertained to the stability of the model and the
231 second the effect of each treatment against the control. For both questions, a step-wise nested
232 modelling approach was taken where after each step a p-value from the likelihood ratio-test was
233 reported.

234

235 For model stability we first analysed the technical replicates and assumed they are independent from
236 each other; that is, we ignored which biological replicate and which bacteria the technical replicate
237 was from. We then assessed the importance of knowing which biological replicate the technical
238 replicates belonged to. The final step assessed the importance of knowing which bacteria the
239 biological replicate belonged to. For the assessment of treatment effect, the model stability data
240 (control data) was pooled with the treated data for each treatment. This pooled data was first
241 analysed by ignoring whether the data was from treated or control group. We then assessed the
242 importance of knowing for which time-series belonged to the control versus treated group. For each
243 treatment we reported the size of the effect compared to control as a % drop with 95% confidence
244 intervals. All data was Log_{10} transformed for the analysis and was conducted in Rv3.1.1 using the
245 *nlme* library.

246

247 **Results**

248 **Developing, Characterising and Optimising the Basally Perfused Model**

249 ***Relative fitness assay***

250 The growth of wound bacteria in binary culture was assessed using monoacetate filters and SFW in a
251 competition assay. A relative fitness close to 1.0 was achieved for all combinations (Table 1). Values

below 1.0 would indicate that the bacterium was less competitive than its congener. Values above 1.0 indicate the advantageous growth of the organism in co-culture, at the cost of growth of the other organism. A small reduction in the relative fitness was observed for *B. fragilis* when combined with all organisms. This reduction was however interpreted as a feature of the growth of this bacterium in relative fitness model due to the requirement for anaerobiosis. Overall data indicate that stable maintenance of the consortium was achievable.

258

Inoculation and population stability

Using a sequential inoculation approach, defined communities were established within 24 h after the final inoculation phase, with each individual species maintaining dynamic stability for a further 8 d (Figure 3). Bacterial populations obtained an overall mean density of *c.* 6 Log₁₀ CFU/mm³ with values of *c.* 6 Log₁₀ CFU/mm³ (0.87 sd) for MRSA, *c.* 5 Log₁₀ CFU/mm³ (0.68 sd) for *S. pyogenes*, *c.* 8 Log₁₀ CFU/mm³ (1.07 sd) for *P. aeruginosa* and *c.* 6 Log₁₀ CFU/mm³ (0.74 sd) for *B. fragilis*.

265

Microbial population stability within biofilm models

The step-wise inclusion of each source of variability in the analysis of the average bacterial count over time for the Model Stability data set can be seen in Table 2. Data suggests that knowing which technical replicate is associated with which biological replicate is important and subsequently knowing which biological replicate belongs to which bacteria is also important. Overall this suggests that the within bacteria variability is lower than the between bacteria variability which implies the experimental system is stable in that it can distinguish between different bacteria.

273

Homogeneity of microbial populations across filters

To assess the homogeneity of population densities in each 5mm x 5mm monoacetate filters were relative to each other at the same time point, communities were grown to a specific timed endpoint

277 and viable counts performed. Table 3 shows the mean, maximum, minimum and standard deviations
278 of viable counts from four filters at each time point. Statistical significance was determined using
279 analysis of variance (ANOVA) combined with post-hoc analysis of least significant difference
280 (LSD) test to determine significant difference. No significant difference was found between viable
281 counts of MRSA, *S. pyogenes*, *P. aeruginosa* or *B. fragilis* between the filters on each day sampled.
282 The basal perfusion model was validated longitudinally and cross-sectionally; a dynamic steady state
283 was achieved up to 8 d in independent runs and comparable populations were obtained between
284 filters harvested at the same time point.

285

286 **Antimicrobial exposure**

287 ***Minimum inhibitory and minimum bactericidal concentrations***

288 Data in Table 4 show the minimum inhibitory and minimum bactericidal concentrations for
289 povidone-iodine, PHMB and silver acetate determined for each organism. All MICs were lower than
290 selected for assessment in the biofilm model, which represent concentrations typically used in topical
291 wound treatments (Bolton 2006, Burks 1998, Butcher 2012, Goldenheim 1993).

292

293 ***Antimicrobial effects on four-species biofilm consortia***

294 The impact of the exposure regimens on the individual populations in the bacterial consortia varied
295 considerably (Figures 4-6). For both PHMB and povidone-iodine, populations of Group A
296 *Streptococcus* became undetectable after dosing, with communities of MRSA and *B. fragilis* also
297 significantly affected, although viable populations of these organisms were detected with increasing
298 frequency (across replicate systems) towards the later phases of dosing in replicated experiments
299 (day 4 onwards). Data for silver acetate dosing show that overall bacterial viability for *P. aeruginosa*
300 remained largely unaffected, with cell counts remaining relatively stable for the remainder of the
301 model runs. There was however, considerable reduction in the viable populations of MRSA, Group

302 A *Streptococcus* and *B. fragilis* 24 h after dosing began but the detectable populations stayed
303 relatively stable for the remainder of the study albeit at a numerically lower density. The overall rank
304 order of effectiveness for the antimicrobials was povidone-iodine > PHMB > silver acetate. The
305 analysis of the effect of each treatment on the average bacterial count over time can be seen in Table
306 5. The magnitude of the treatment effect on the bacterial population varies across the treatments. The
307 largest reduction in bacterial count was seen for povidone-iodine (57%) followed by PHMB (44%)
308 and then silver acetate (27%).

309

310 ***Biofilm imaging***

311 ESEM revealed afferent bacteria and microcolonies of proportions, along with and putative biofilm
312 EPS throughout inoculated filters (Figure 7 b-d). Comparisons with the negative control filter
313 (Figure 7a) indicate that the microcolonies and putative EPS observed were the result of inoculation
314 and environmental conditions of the model and were not an artifact of the filter structure or media.

315

316 **Discussion**

317 There is considerable interest in the development of effective regimens for the management of
318 biofilms within complex wounds. *In vivo* and *in vitro* biofilm models have previously been used as
319 research tools in this field. An issue of concern with biofilm models however is the ability to
320 maintain populations comprising bacteria with distinct oxygen requirements in a system that
321 facilitates the effects of antimicrobial compounds to be determined. With the broad aim of providing
322 a platform in which this could be explored, an *in vitro* wound biofilm model was developed that
323 reproduces the basolateral nutrient delivery of wounds, and which supports the growth of a defined
324 consortium comprising of organisms of interest in wounds with distinct antimicrobial
325 susceptibilities, general physiology and growth requirements.

326 Before designing the wound basal perfusion model, currently available model systems were
327 assessed for their potential use. These included the Multiple Sorbarod Device (Ledder et al. 2006)
328 and a Flat-Bed Perfusion Biofilm model (Thorn and Greenman 2009). To develop a model suitable
329 for the required application, selected characteristics of these systems were adopted, including the use
330 of filters as substrata and a nutrient delivery system which supplied nutrient media to the basally.
331 The developed system combines a continuous perfusion, similar to exudate flow in a wound, with
332 eight sampling positions to support the continuous assessments of a treatment over time or a single
333 treatment point with multiple replicates.

334 In preliminary investigations, when models were concomitantly inoculated with MRSA, *S.*
335 *pyrogens*, *P. aeruginosa* and *B. fragilis*, all species were initially detectable. However, after 24 h,
336 MRSA and *S. pyogenes* rapidly declined and became undetectable, whilst populations of *P.*
337 *aeruginosa* and *B. fragilis* expanded. To determine if the test bacteria could in principle grow in
338 consortia under optimised conditions, relative fitness assays were performed in which the test
339 organisms were grown together in all binary combinations with no evidence of specific growth
340 inhibition. These data supported the initial observations of 24 h of growth in a prototype system,
341 where viable Gram-positive organisms were detectable within 24 h of inoculation. Based on these
342 observations a sequential inoculation procedure was developed in which MRSA and *S. pyogenes*
343 were established prior to the introduction of the Gram-negative organisms including the obligate
344 anaerobe, *Bacteroides fragilis* after 24 h. Using this approach, all four organisms became established
345 within defined species wound-type biofilms for up to 8 d.

346 The inclusion of eight sampling positions in the biofilm model facilitated the collection of
347 multiple baseline and treatment samples to monitor stability and reproducibility. The similarity in
348 population densities between replicated model runs indicates that baseline values and daily sampling
349 would provide a suitable platform through which to test the efficacy of antimicrobial therapies such
350 as wound dressings over a 7 d period.

351 Three topical wound treatments with distinct modes of action were investigated; silver
352 acetate, povidone-iodine and polyhexamethylene biguanide (PHMB) against the four-species
353 consortium. For povidone-iodine and PHMB, concentrations of 10% and 0.5% (respectively) were
354 selected based upon concentrations typically used in topical wound treatments (Burks 1998, Butcher
355 2012, Goldenheim 1993). Defining a relevant concentration for silver was however, more
356 complicated, with previous reports of various silver compounds such as silver acetate, silver nitrate
357 and nanoparticles being used at various concentrations in a range of commercially available wound
358 dressings (Bolton 2006, Fong and Wood 2006). Following a literature review, silver acetate was
359 selected and used at the highest soluble concentration achievable in the growth medium, which was
360 greater than the MIC for silver acetate for each test bacterium.

361 Based on replicated determinations, the rank order of effectiveness for the antimicrobials in
362 planktonic susceptibility tests was (highest to lowest) silver acetate > povidone iodine > PHMB. For
363 biofilm communities however, it was povidone-iodine > PHMB > silver acetate. Possible reasons for
364 this disparity include the fact that i) the mechanisms that underlie antimicrobial tolerance with
365 biofilms are partly distinct from those that are assessed in planktonic susceptibility tests and ii) that
366 povidone iodine was applied to the biofilms at 10% (w/v), polyhexamethylene biguanide at 0.5% and
367 silver acetate at 0.05% (w/v), therefore planktonic susceptibility data (MIC and MBC) cannot
368 necessarily be used to predict the outcome of biofilm exposure. These two considerations are of
369 relevance in the assessment of the effectiveness of antimicrobials for wound care, reinforcing the
370 need to consider the biofilm phenotype in antimicrobial tests and to consider that the concentrations
371 of distinct antimicrobial compounds used in wound care vary for reasons that include formulation,
372 toxicity and regulation.

373 For data generated using the wound model, both povidone-iodine and PHMB had the greatest
374 impact on MRSA, *B. fragilis* and Group A *Streptococcus*, with the latter undetectable in both the
375 povidone-iodine and PHMB dosed systems from second day of dosing. For povidone-iodine, PHMB

376 and silver acetate-dosed systems; *P. aeruginosa* was detected throughout, with silver acetate being
377 the least effective of the antimicrobials tested against this bacterium. In some cases, following the
378 initial inactivation of bacteria, partial recovery of cell counts was observed during continual
379 exposure to antimicrobials. This was apparent following initial exposure and was notable for *P.*
380 *aeruginosa* during exposure to povidone-iodine and PHMB and for MRSA during exposure to
381 PHMB and silver acetate.

382 When a microbial community is exposed to an antimicrobial agent, organisms will generally
383 be inactivated in order of their susceptibility. The well documented tolerance of biofilms towards
384 antimicrobials may increase this effect, since it is less likely that a biofilm community will be
385 completely inactivated than the same organisms growing in dispersed, planktonic form. An additional
386 consideration is that the tolerance conferred by biofilm growth may be greater for some organisms
387 than for others. In the current context, the recalcitrance of *P. aeruginosa* (Pedersen, 1992; Gilbert et
388 al., 2002; Walters et al., 2003), its tendency to form biofilms, and the recalcitrance of those biofilms
389 once developed, has been widely reported. Thus, the relative susceptibility of bacteria observed in
390 the current study, and the survival of *P. aeruginosa* in exposed biofilms could be largely predicted
391 based on known features of this bacterium. The observed inactivation kinetics could be explained by
392 phenotypic adaptation within the biofilm, whilst the varied tolerance to antimicrobial exposure
393 observed in the basal perfusion model against the three compounds resembles that previously
394 reported by Forstner et al. (2013) using a porcine wound model. Since distinct bacteria often
395 exhibited distinct antimicrobial susceptibility profiles to antiseptics as well as to antibiotics, such
396 variable antibacterial effects may be an important but relatively little studied variable in wound
397 antisepsis (Davis et al. 2006, James et al. 2008, Roy et al. 2014, Saye 2007).

398 In conclusion, the primary objective of the current investigation was to develop an *in vitro*
399 biofilm model which would i) support the growth of a mixed species consortia; ii) could be used
400 model antisepsis and iii) would facilitate the establishment of replicated biofilm consortia for

temporal studies. A defined species biofilm consortium containing aerobic, facultative and anaerobic species was grown reproducibly using the basally perfused model. The effect of topical wound antimicrobials was examined and variable susceptibility in the four test bacteria was evident in biofilm consortia despite concentrations used being considerably higher than planktonic susceptibility, a phenomenon often associated with biofilms.

406

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409 Materials, University of Manchester for access to the ESEM and assistance with the imaging by Dr.
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411

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415

416 **References**

417

418 Barnea Y, Weiss J, Gur E. 2010. A review of the applications of the hydrofiber dressing with silver
419 (Aquacel Ag) in wound care. *Therapeutics and Clinical Risk Management*. 6:21-27.

420

421 Bjarnsholt T, Kirketerp-Moller K, Jensen PO, Madsen KG, Phipps R, Krogfelt K, Hoiby N, Givskov
422 M. 2008. Why chronic wounds will not heal: a novel hypothesis. *Wound Repair Regen*. 16:2-10.

423 Bolton L. 2006. Are silver products safe and effective for chronic wound management? *J Wound
424 Ostomy Continence Nurs*. 33:469-477.

425 Bradley M, Cullum N, Nelson EA, Petticrew M, Sheldon T, Torgerson D. 1999. Systematic reviews
426 of wound care management: (2). Dressings and topical agents used in the healing of chronic wounds.
427 *Health Technol*. 3:1 - 35.

428 Burks RI. 1998. Povidone-iodine solution in wound treatment. *Physical therapy*. 78:212-218.

429 Butcher M. 2012. PHMB: an effective antimicrobial in wound bioburden management. *Br J Nurs*.
430 11;21:S16, S18-21.

431 Claessen D, Rozen DE, Kuipers OP, Sogaard-Andersen L, van Wezel GP. 2014. Bacterial solutions
 432 to multicellularity: a tale of biofilms, filaments and fruiting bodies. *Nat Rev Microbiol.* 12:115-124.
 433 Epub 2014/01/05.

434 Costerton JW. 1999. Introduction to biofilm. *Int J Antimicrob Agents.* 11:217-221.

435 Davis SC, Martinez L, Kirsner R. 2006. The diabetic foot: the importance of biofilms and wound bed
 436 preparation. *Curr Diab Rep.* 6:439-445.

437 Fong J, Wood F. 2006. Nanocrystalline silver dressings in wound management: a review.
 438 *International Journal of Nanomedicine.* 1:441-449.

439 Forstner C, Leitgeb J, Schuster R, Dosch V, Kramer A, Cutting KF, Leaper DJ, Assadian O. 2013.
 440 Bacterial growth kinetics under a novel flexible methacrylate dressing serving as a drug delivery
 441 vehicle for antiseptics. *Int J Mol Sci.* 14:10582-10590.

442 Gilbert P, Maira-Litran T, McBain AJ, Rickard AH, Whyte FW. 2002. The physiology and
 443 collective recalcitrance of microbial biofilm communities. *Adv Microb Physiol.* 46:202-256.

444 Goldenheim PD. 1993. An appraisal of povidone-iodine and wound healing. *Postgraduate Medical*
 445 *Journal.* 69 Suppl 3:S97-105.

446 Hall-Stoodley L, Costerton JW, Stoodley P. 2004. Bacterial Biofilms: from the Natural Environment
 447 to Infectious Diseases. *Nat Rev Microbiol.* 2:95-108.

448 Hausner M, Wuertz S. 1999. High rates of conjugation in bacterial biofilms as determined by
 449 quantitative in situ analysis. *Appl Environ Microbiol.* 65:3710-3713.

450 Hill KE, Malic S, McKee R, Rennison T, Harding KG, Williams DW, Thomas DW. 2010. An in
 451 vitro model of chronic wound biofilms to test wound dressings and assess antimicrobial
 452 susceptibilities. *J Antimicrob Chemother.* 65:1195-1206.

453 Hodgson AE, Nelson SM, Brown MR, Gilbert P. 1995. A simple *in vitro* model for growth control
 454 of bacterial biofilms. *J Appl Bacteriol.* 79:87-93.

455 Humphreys G, Lee GL, Percival SL, McBain AJ. 2011. Combinatorial activities of ionic silver and
 456 sodium hexametaphosphate against microorganisms associated with chronic wounds. *J Antimicrob*
 457 *Chemother.* 66:2556-2561.

458 James G, Swogger E, Wolcott R, deLancey-Pulcini E, Secor P, Sestrich J, Costerton JW, Stewart PS.
 459 2008. Biofilms in chronic wounds. *Wound Repair and Regeneration.* 16:37-44.

460 Kanno E, Toriyabe S, Zhang L, Imai Y, Tachi M. 2009. Biofilm formation on rat skin wounds by
 461 *Pseudomonas aeruginosa* carrying the green fluorescent protein gene. *Experimental Dermatology.*
 462 19:154-156.

463 Ledder RG, Gilbert P, Pluen A, Sreenivasan PK, De Vizio W, McBain AJ. 2006. Individual
 464 microflora beget unique oral microcosms. *J Appl Microbiol.* 100:1123-1131.

465 Lenski RE, Rose MR, Simpson SC, Tadler SC. 1991. Long-term experimental evolution in
 466 *Escherichia coli*. 1. Adaptation and divergence during 2,000 generations. *American Society of*
 467 *Naturalists.* 1315-1341.

468 Lipp C, Kirker K, Agostinho A, James G, Stewart P. 2010. Testing wound dressings using an in vitro
469 wound model. *Journal of Wound Care*. 19:220-226.

470 McCarthy H, Rudkin JK, Black NS, Gallagher L, O'Neill E, O'Gara JP. 2015. Methicillin resistance
471 and the biofilm phenotype in *Staphylococcus aureus*. *Frontiers in Cellular and Infection*
472 *Microbiology*. 5:1.

473 Neut D, Tijdens-Creusen EJ, Bulstra SK, van der Mei HC, Busscher HJ. 2011. Biofilms in chronic
474 diabetic foot ulcers-a study of 2 cases. *Acta Orthop*. 82:383-385.

475 Oates A, Bowling FL, Boulton AJM, Bowler PG, Metcalf DG, McBain AJ. 2014. The Visualization
476 of Biofilms in Chronic Diabetic Foot Wounds Using Routine Diagnostic Microscopy Methods.
477 *Journal of Diabetes Research*. 2014:8-16.

478 Oates A, McBain AJ. 2016. Growth of MRSA and *Pseudomonas aeruginosa* in a fine-celled foam
479 model containing sessile commensal skin bacteria. *Biofouling*. 32:25-33. Epub 2016/01/05.

480 Ohadian Moghadam S, Pourmand MR, Aminharati F. 2014. Biofilm formation and antimicrobial
481 resistance in methicillin-resistant *Staphylococcus aureus* isolated from burn patients, Iran. *Journal of*
482 *infection in developing countries*. 8:1511-1517.

483 Pedersen SS. 1992. Lung infection with alginate-producing, mucoid *Pseudomonas aeruginosa* in
484 cystic fibrosis. *APMIS Supplementum*. 28:1-79.

485 Ramos AN, Peral MC, Valdez JC. 2010. Differences between *Pseudomonas aeruginosa* in a clinical
486 sample and in a colony isolated from it: Comparison of virulence capacity and susceptibility of
487 biofilm to inhibitors. *Comparative Immunology, Microbiology and Infectious Diseases*. 33:267-275.

488 Rochex A, Lebeault JM. 2007. Effects of nutrients on biofilm formation and detachment of a
489 *Pseudomonas putida* strain isolated from a paper machine. *Water Research*. 41:2885-2892.

490 Roy S, Elgharably H, Sinha M, Ganesh K, Chaney S, Mann E, Miller C, Khanna S, Bergdall VK,
491 Powell HM, et al. 2014. Mixed-species biofilm compromises wound healing by disrupting epidermal
492 barrier function. *The Journal of Pathology*. 233:331-343.

493 Rozen DE, McGee L, Levin BR, Klugman KP. 2007. Fitness costs of fluoroquinolone resistance in
494 *Streptococcus pneumoniae*. *Antimicrob Agents Chemother*. 51:412-416.

495 Saye DE. 2007. Recurring and antimicrobial-resistant infections:considering the potential role of
496 biofilms in clinical practice. *Ostomy Wound Manage*. 53:46-48.

497 Scali C, Kunitomo B. 2013. An Update on Chronic Wounds and The Role of Biofilms. *Journal of*
498 *Cutaneous Medicine and Surgery*. 17:371-376.

499 Schierle CF, De la Garza M, Mustoe TA, Galiano RD. 2009. Staphylococcal biofilms impair wound
500 healing by delaying reepithelialization in a murine cutaneous wound model. *Wound Repair and*
501 *Regeneration*. 17:354-359.

502 Sorensen SJ, Bailey M, Hansen LH, Kroer N, Wuertz S. 2005. Studying plasmid horizontal transfer
503 in situ: a critical review. *Nat Rev Microbiol*. 3:700-710.

504 Stewart PS, Costerton JW. 2001. Antibiotic resistance of bacteria in biofilms. *Lancet*. 358:135-138.

505 Sun Y, Dowd SE, Smith E, Rhoads DD, Wolcott RD. 2008. In vitro multispecies Lubbock chronic
506 wound biofilm model. *Wound Repair and Regeneration*.16:805-813.

507 Thorn RMS, Greenman J. 2009. A novel in vitro flat-bed perfusion biofilm model for determining
508 the potential antimicrobial efficacy of topical wound treatments. *Journal of Applied Microbiology*.
509 107:2070-2079.

510 Walters MC, 3rd, Roe F, Bugnicourt A, Franklin MJ, Stewart PS. 2003. Contributions of antibiotic
511 penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa*
512 biofilms to ciprofloxacin and tobramycin. *Antimicrob Agents Chemother*. 47:317-323.

513 Watanabe K, Teramoto M, Futamata H, Harayama S. 1998. Molecular detection, isolation, and
514 physiological characterization of functionally dominant phenol-degrading bacteria in activated
515 sludge. *Appl Environ Microbiol*. 64:4396-4402.

516 Werthén M, Henriksson L, Jensen PØ, Sternberg C, Givskov M, Bjarnsholt T. 2010. An in vitro
517 model of bacterial infections in wounds and other soft tissues. *APMIS*. 118:156-164.

518 Wilkinson HN, McBain AJ, Stephenson C, Hardman MJ. 2016. Comparing the Effectiveness of
519 Polymer Debriding Devices Using a Porcine Wound Biofilm Model. *Adv Wound Care (New*
520 *Rochelle)*. 5:475-485.

521 Wolcott RD, Rhoads DD. 2008. A study of biofilm-based wound management in subjects with
522 critical limb ischaemia. *J Wound Care*. 17:145-148, 150-142, 154-145.

523 Woods J, Boegli L, Kirker KR, Agostinho AM, Durch AM, Delancey Pulcini E, Stewart PS, James
524 GA. 2012. Development and application of a polymicrobial, in vitro, wound biofilm model. *J Appl*
525 *Microbiol*. 112:998-1006.

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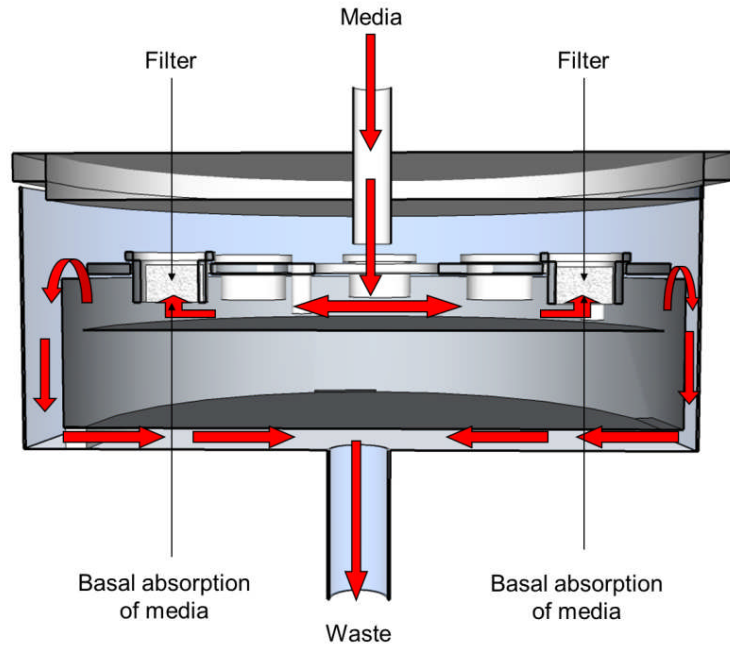
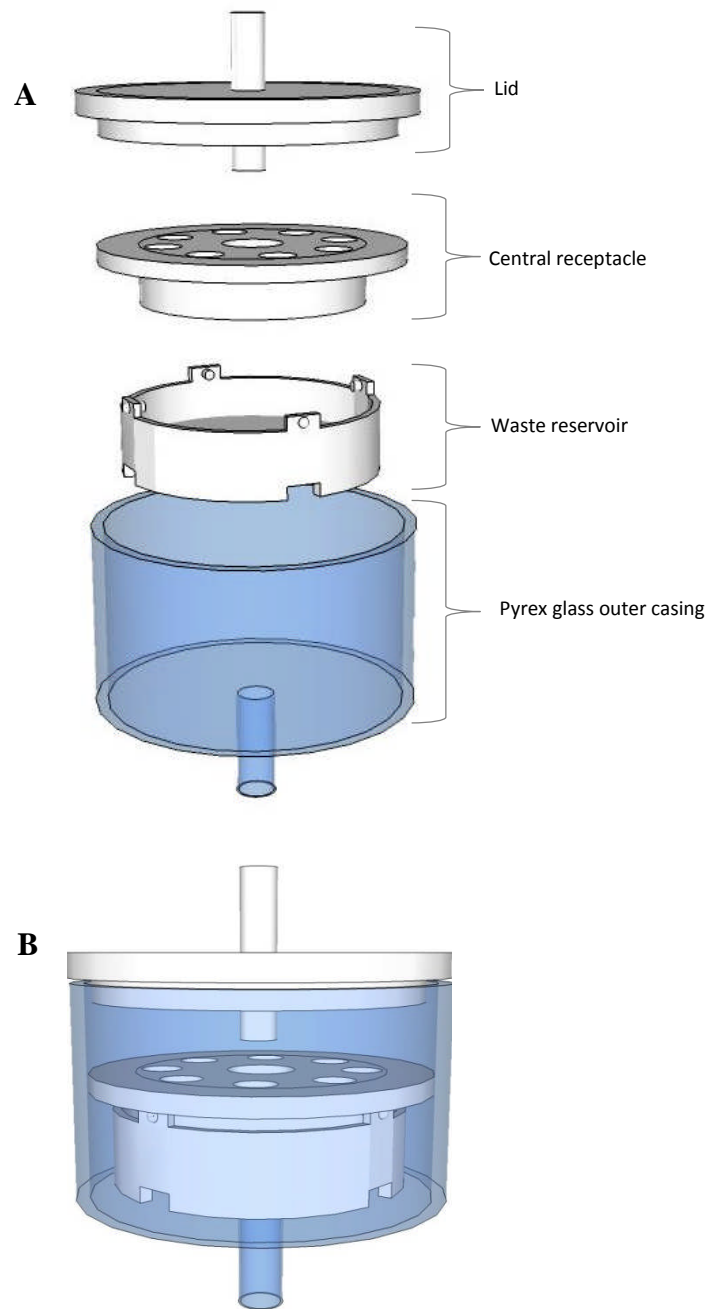


Figure 1. A diagrammatical cross-sectional view of the basal perfusion model illustrating the media delivery and waste outflow. The filters on which biofilms are grown are 5mm x 5mm in size and are placed within the individual holes (8 in total) of the central fluid basin. The upper section and lower section of these individual holes are separated by a small rim/ledge upon which the filter rest.

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Figure 2. A diagrammatical view of the individual components (A) and combined (B) components of the basal perfusion wound model.

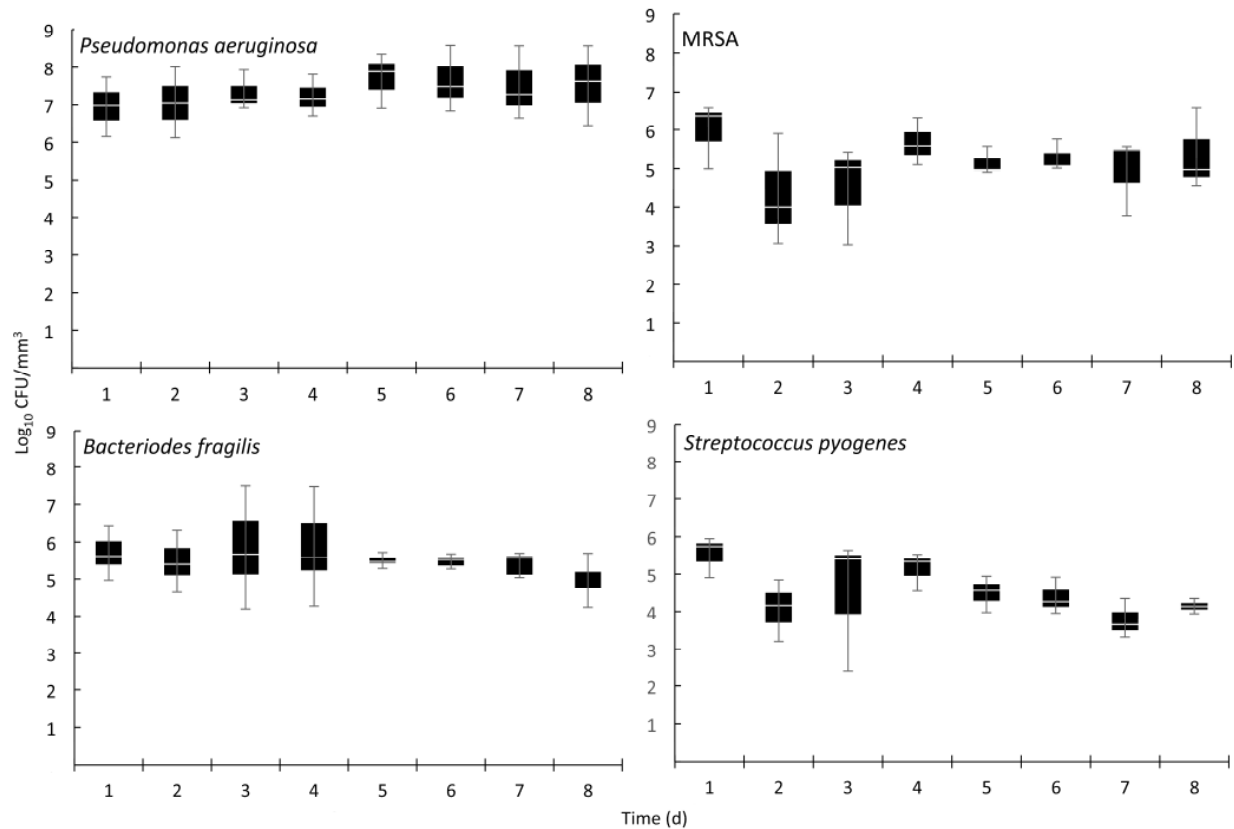


Figure 3. Population densities of the four species wound consortia grown and maintained for 8 d in the three independent basal perfusion models. The boxes represent the interquartile range with the lower and upper boundaries of the boxes representing quartiles 1 and 3; the central line is the median. The whiskers represent the maximum and minimum values. Data represent mean and ranges for three independent experiments. Detection threshold, <1.0 Log₁₀ CFU/mm³

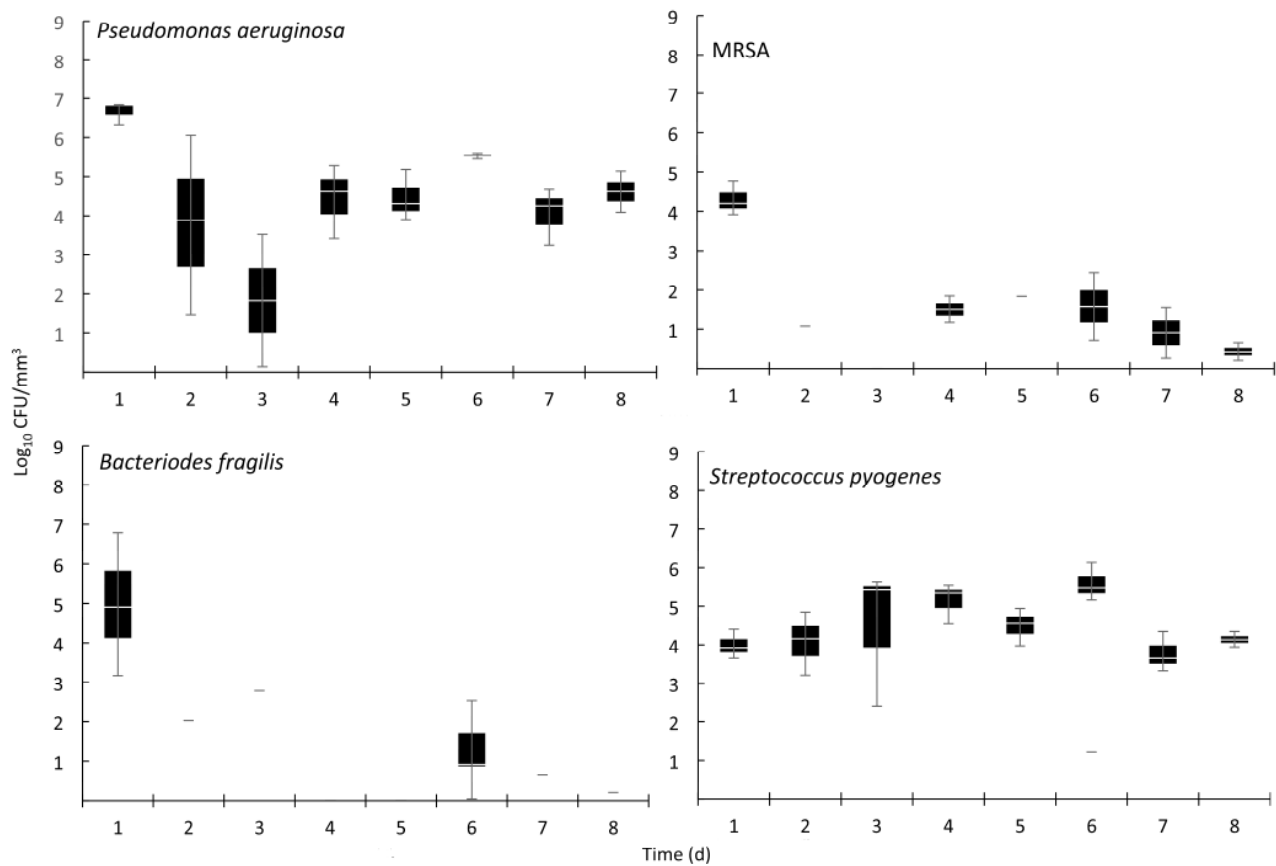


Figure 4. Population densities the four species wound consortia grown in the basal perfusion models and exposed to 10% povidone-iodine. Single horizontal lines indicate viable count data for one replicate above the detection threshold. See legend to Figure 3.

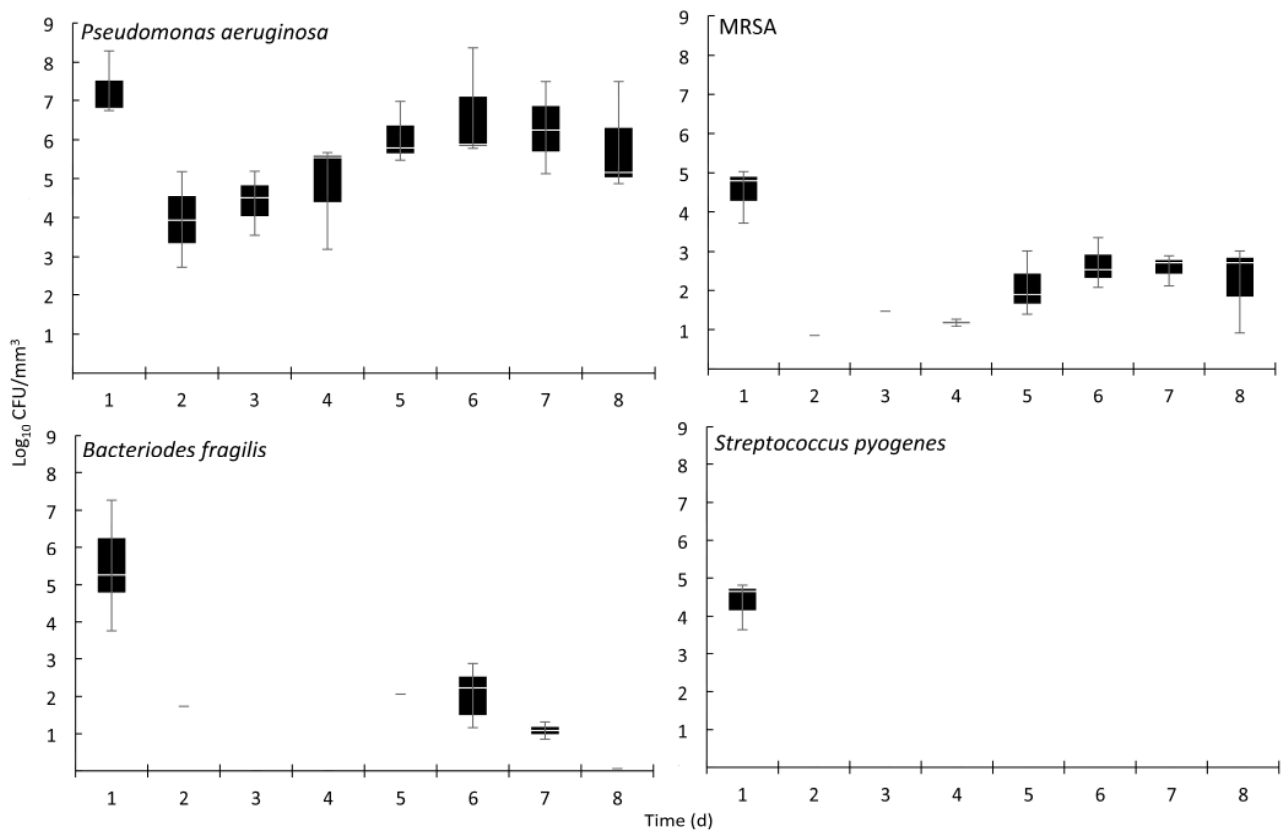
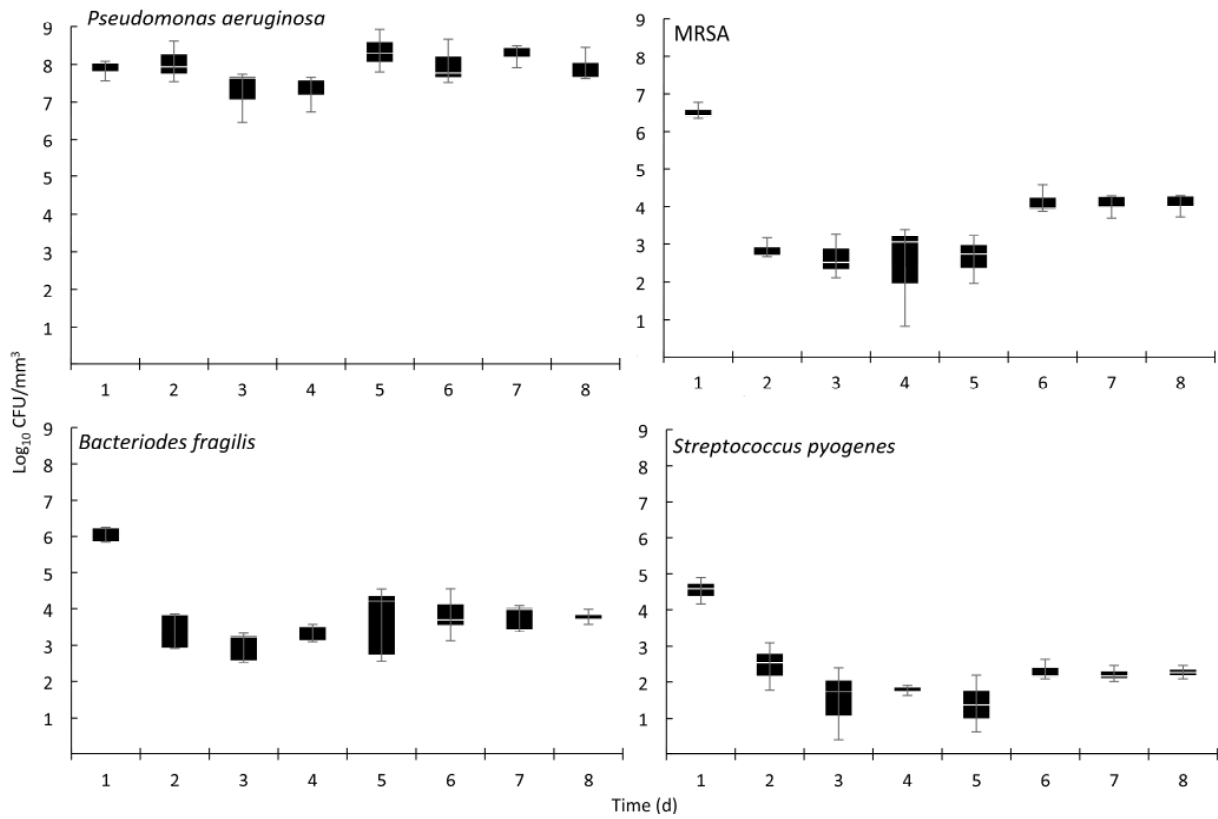


Figure 5. Population densities the four species wound consortia grown in the basal perfusion models and exposed to 0.5% PHMB. See legend to Figure 3.

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Figure 6. Population densities the four species wound consortia grown in the basal perfusion models and exposed to 0.05 % silver acetate. See legend to Figure 3.

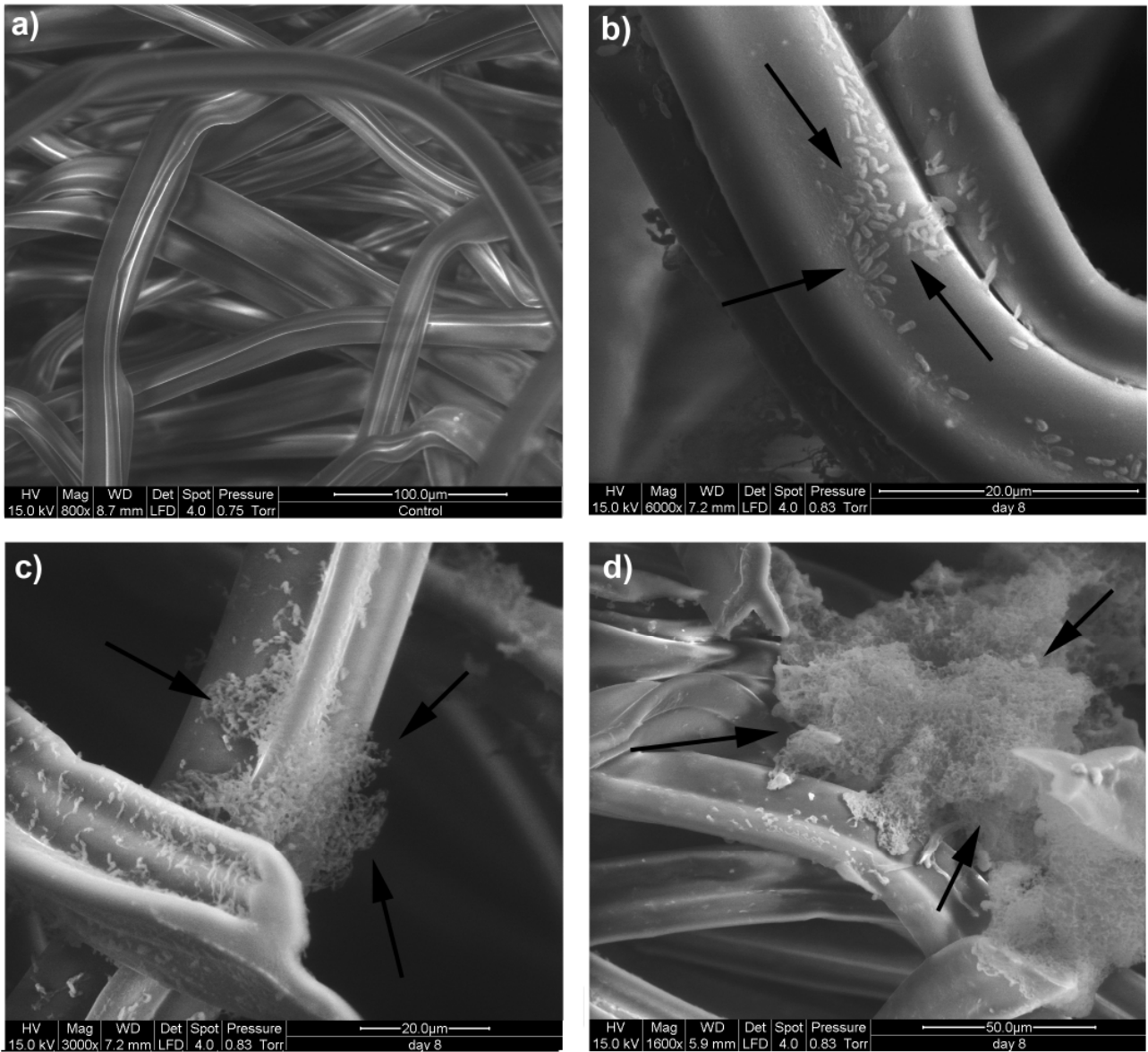


Figure 7. ESEM images showing (a) an uninoculated filter; (b) adherent bacteria (mainly rod morphology); (c) a microcolony and (d) a larger colony/biofilm of coccoid bacteria on filter samples taken from inoculated models.

Table 1. The relative fitness of organisms in the modified competition assay

Relative fitness of → versus ↓	Bacterium			
	MRSA	<i>S. pyogenes</i>	<i>P. aeruginosa</i>	<i>B. fragilis</i>
MRSA	na	1.05 (0.02)	0.96 (0.09)	0.83 (0.03)
<i>S. pyogenes</i>	0.95 (0.04)	na	1.13 (0.04)	0.84 (0.03)
<i>P. aeruginosa</i>	1.04 (0.14)	0.89 (0.07)	na	0.78 (0.15)
<i>B. fragilis</i>	1.21 (0.05)	1.19 (0.10)	1.28 (0.15)	na

na, not applicable. Data are means (SEM) of three separate experiments.

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640 **Table 2.** The goodness of fit value, -2LL, for each iteration of the model, together with p-value from
641 the likelihood ratio test for model stability assays

Replicate type	-2LL (p-value)
Technical Replicates (1)	567
Technical Replicates in Biological replicates (2)	542 (2 v 1: $p < 0.001$)
Technical Replicates in Biological replicates in type of Bacteria (3)	533 (3 v 2: $p = 0.004$)

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647 **Table 3.** The mean, maximum, minimum and standard deviation of population densities from four separate mono-acetate filters
648 sampled at the same time point

Day	Bacterium															
	MRSA				<i>S. pyogenes</i>				<i>P. aeruginosa</i>				<i>B. fragilis</i>			
	Mean	Max	Min	Stdev	Mean	Max	Min	Stdev	Mean	Max	Min	Stdev	Mean	Max	Min	Stdev
1	7.72	7.93	7.52	0.17	8.58	9.10	8.25	0.38	9.52	9.60	9.43	0.08	9.32	9.35	9.26	0.04
2	7.73	7.84	7.68	0.07	7.62	7.73	7.49	0.11	9.53	9.62	9.43	0.10	9.28	9.32	9.23	0.04
3	7.31	7.59	7.11	0.22	7.42	7.60	7.24	0.17	9.45	9.47	9.43	0.02	9.12	9.17	9.04	0.06
4	7.28	7.38	7.19	0.09	7.62	7.76	7.50	0.11	9.31	9.35	9.25	0.04	9.24	9.39	9.07	0.13
5	7.34	7.52	7.24	0.13	7.08	7.22	6.96	0.11	9.26	9.42	9.05	0.16	8.92	9.09	8.65	0.19
6	7.50	7.68	7.26	0.20	7.00	7.20	6.81	0.16	9.40	9.48	9.36	0.05	9.13	9.57	8.94	0.30
7	7.06	7.68	6.46	0.66	6.99	7.24	6.77	0.20	9.37	9.39	9.35	0.02	8.07	8.22	7.93	0.12
8	6.45	6.55	6.23	0.15	7.62	8.08	6.96	0.53	9.46	9.51	9.40	0.06	7.98	8.04	7.92	0.06

649 Data are Log₁₀ CFU per mm³.

Table 4. Planktonic susceptibility determined in simulated wound fluid

Bacterium	Planktonic susceptibility					
	MIC			MBC		
	PHMB	Silver acetate	Povidone-iodine	PHMB	Silver acetate	Povidone-iodine
MRSA	7.94 (2.75)	0.08	5210 (1800)	101 (44.01)	1.25	25000
<i>S. pyogenes</i>	4.76	0.02	490 (4100)	22.23 (14.55)	0.26 (0.09)	20839 (7220)
<i>P. aeruginosa</i>	152	0.04	8330 (3610)	2032 (704)	1.25	25000
<i>B. fragilis</i>	6.35 (2.75)	0.07 (0.02)	3130	25.00	nd	25000

All concentrations are mg/L. Standard deviations are given in parenthesis, nd, not determined (>1.25mg/l).
Data are means from three separate determinations.

Table 5. The difference in -2LL between each treatment, control and model stability showing p-values derived from the likelihood ratio test for the treatment assays

Treatment	-2LL Drop (p-value)	% Relative Drop (95% CI)
Povidone-iodine	46 (p<0.001)	57 (51, 63)
PHMB	27 (p<0.001)	44 (35, 53)
Silver acetate	12 (p<0.001)	27 (15, 39)

CI, confidence interval.